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"SAFE AND EFFECTIVE STIMULATION OF NEURAL TISSUE"

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This QPR is being sent to you before it has been reviewed by the staff of the Neural Prosthesis Program.

Recovery of Sciatic Nerve Following Damaging Electrical Stimulation

ABSTRACT

Stimulation of the sciatic nerve of the cat using continuous, high frequency, high amplitude electrical parameters for 8 hours results in early axonal degeneration (EAD) in up to 4.6% of the fibers as quantified by the use of computerized image analysis. Light and electron microscopy was used to demonstrate the characteristics and fate of the axonal degeneration at 7 to 60 days following stimulation. The axonal injury was irreversible in most if not all cases and advanced axonal degeneration (AAD) persisted even to 60 days following the stimulation. Evidence of neuronal loss was observed in some nerves with very little remyelination or other evidence of regeneration of damaged axons. No neurological deficits were observed in any of the animals.

INTRODUCTION

The present study is a continuation of our efforts to develop safe and efficient stimulation protocols for functional electrical stimulation (FES) applications for the neurologically handicapped. Previous studies conducted in our laboratory have demonstrated the importance and interaction of several parameters in the induction of injury to peripheral nerves during eight hours of continuous electrical stimulation. These included total duration of the stimulation, stimulus frequency, stimulus duty cycle, stimulus pulse amplitude (Agnew et al, 1989, McCreery et al, 1995) and the effects of a "long" vs. "short" pulse, i.e., with and without an interpulse delay (McCreery et al, 1992).

Prolonged, high frequency electrical stimulation of a peripheral nerve induces a characteristic type of neural injury, early axonal degeneration (EAD), that consists of a collapse of the myelin into the axoplasmic space (Agnew et al, 1989). The extent of the

reversibility and remyelination associated with the electrically-induced injury has not been determined. In the present study, the animals were sacrificed at poststimulation intervals varying from seven days to 60 days, to determine the fate and extent of the recovery of damaged myelinated fibers. In addition to light and electron microscopic evaluation, we have also quantitated changes in the nerve by the use of computerized morphometric analysis.

METHODS

Electrode: The HMRI bidirectional, helical stimulating electrode array was used in this study. The electrode array consists of two platinum bands, 1.0 mm in width and approximately 5 mm in length. The platinum bands are supported on their outer circumference by a matrix of silicone elastomer, which uncoils slightly as the array is installed on the nerve. Thus the electrodes are self-sizing and completely circumneural so that the nerve is excited from around its entire perimeter.

Surgical Technique: Adult cats of either sex were anesthetized, and using aseptic surgical techniques, a modified Cannon percutaneous connector was attached to the skull using stainless steel screws and cranioplasty. This connector provided an interface for both stimulating and recording leads. Two stimulating and recording electrodes with attached cables were then tunneled subcutaneously to the lumbosacral area. In the lumbosacral region, one recording electrode was fixed to the subcutaneous fascia at approximately L-5, and the counter recording electrode was fixed to the midline fascia 3-4 cm caudal to the first. The two stimulating leads and bipolar electrodes were tunneled to the area of the left and right sciatic nerves, following the sciatic notches alongside the nerves. The sciatic nerves were exposed using a lateral approach paralleling the biceps femoris muscle. A 2-3 cm length of the sciatic nerve was elevated with a moistened flat rubber band to facilitate installation of the stimulating array which was accomplished with the aid of a modified bayonet

forceps which spreads the coils of the array. The cable to the array was sutured to the epineurium by three 7-0 silk sutures.

Stimulation And Recording Protocols: Three or four weeks following implantation of the electrodes, the cats were anesthetized by continuous intravenous Propofol and both sciatic nerves were stimulated continuously, for eight hours, with sufficient amplitude to induce axonal injury. The stimulus waveform was a charge-balanced, controlled current, biphasic pulse-pair at a frequency of 50 Hz. Each phase of the pair was 100 μsec in duration, with a 400 μsec delay between the first and second phases. This waveform excites both large and small myelinated fibers in the nerve (Gorman and Mortimer, 1983; van der Honert and Mortimer, 1979; McCreery et al, 1992). Just before the start of the eight hours of continuous stimulation, we measured the recruitment characteristics of the averaged compound action potential (ACAP) evoked by the stimulating electrodes and recorded over the spinal cord. In the sciatic nerve of the cat, the first large component (the α component) of the ACAP represents the activity in the alpha and gamma efferent axons and the large afferent axons. To determine the recruitment characteristics of the α component of the ACAP, the nerve was stimulated at several different current amplitudes, and the amplitude of the α component was plotted against the stimulus pulse amplitude. The stimulus current needed to fully recruit the α component was used as a normalizing factor, i.e., the current was expressed as multiples of one full α rather than as μamps. The ACAP was recorded at the end of the eight hours of stimulation and again just before sacrifice of the animal.

At various intervals (7 - 90 days) following stimulation, the animals were anesthetized with Nembutal and perfused via the ascending aorta with ½ strength Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate). The sciatic nerves were resected from the leg and embedded in Polybed plastic (Polysciences). Cross sections cut to a thickness of 1 μm were taken from beneath each electrode, between the electrodes and 1 cm proximal and 1 cm distal to

the electrode array. From these sections, areas of interest were chosen for transmission electron microscopic examinations. The extent and severity of axonal injury was determined by light and electron microscopy. A few specific features of axonal changes (EAD, remyelination and axonal loss) were quantitated by image analysis.

Morphometric Analysis: The details of the computerized image analyses have been presented previously (McCreery et al, 1997). All data acquisition and analyses were implemented using an IBM-compatible computer. For each nerve, 11-13 microscopic fields from the largest fascicles, each spanning approximately 186 x 180 μm, and representing ≈20% - 25% of the cross-sectional area of nerve, were scanned into the computer, using a high-resolution CCD camera (EDC-100, Electrim, Inc.). The number of injured axons was based on counts from sections taken beneath the proximal electrode. The image fields were evenly distributed over the cross-sections of the two largest fascicles.

Each digitized image was processed using a commercial image analysis (IA) program (Global Lab Image, from Data Translation). The Image Analysis Program identifies the profiles of the individual myelinated fibers and computes the cross-sectional area of the myelin and of the axoplasm of each fiber. These tabulated data are then processed further by custom software. Fibers for which the ratio of axoplasm-to-myelin is low ($A < (M - 5.8) / 5$) are classified as having shrunken axonal profiles (SAPs). In this equation, "A" is the area of the axoplasmic profile in μm^2, and "M" is the area of the myelin profile. The SAPs are axons undergoing early axonal degeneration (EAD) or advanced axonal degeneration (AAD), and also, a small number of profiles that happen to be sectioned through the perinodal region where the ratio of axoplasm to myelin is normally low. These comprise about 0.1% of the total. Fibers for which the myelin is abnormally thin ($M < 0.67A$) are classified as hypomyelinated. The criteria for

SAPs and hypomyelinated fibers was derived from data taken from five normal, unstimulated cat sciatic nerves and 11,407 myelinated axons (McCreery et al, 1997).

RESULTS

Clinical Results. For the first 1-2 days following surgery, the cats assumed a crouching posture, presumably due to subcutaneous and muscular trauma from the surgery. There was no indication of any neurological deficit in any of the cats, e.g., loss of placing.

Autopsy Results. A connective tissue (CT) sheath always covered the array and adjacent portions of the sciatic nerve. The whitish, translucent sheath ranged in thickness from 80 to 750 μm but usually was about 300 μm thick. The sheath was easily resected and revealed the underlying, loosely fitting array encircling the nerve (Fig. 1). In a few instances, epineurial hemorrhages were present beneath or adjacent to the electrodes and usually were present immediately around the epineurial sutures. In PN-192, both sciatic nerves were found to be bent at a 135° angle at the proximal electrode. In no instance did the nerves show gross evidence of constriction or other physical force.

Histologic Results. At seven days following intense electrical stimulation (Table I), the most prominent feature of the neural damage was early axonal degeneration (EAD), as we have previously reported (Agnew et al., 1989). This appeared as a "salt and pepper" pattern of dark EAD profiles, interspersed among lighter profiles of normal myelinated axons (Figs. 2A and 2B). As we have shown previously, the EAD is not apparent in the intensely stimulated nerve until seven days after stimulation. The EAD was not always uniformly distributed in all fascicles of the nerve. In some cases heavy EAD was observed in one or two fascicles with sparse distribution in a third, suggesting nonuniform distribution of the current, even though the platinum electrodes were circumferential. Longitudinal sections through the nerve

TABLE I

ANIMAL NO.	PN NO.	NERVE (LEFT OR RIGHT)	I* (μA)	I* (× FULL α)	PERCENT# EAD/AAD	POST-STIM. TIME (DAYS)
1	176	L	2100	3.0	3.5	7
		R	2100	4.0	1.0	
2	169	L	2700	3.0	1.5	7
		R	3000	4.2	4.6	
3	171	L	2400	3.0	3.1	7
		R	2400	3.0	1.4	
4	188	L	3150	4.5	na	21
		R	3150	4.5	na	
5	189	L	3500	4.5	0.4	28
		R	3800	4.5	0.8	
6	192	L	3250	4.5	0.1	36
		R	2650	4.5	0.7	
7	179	L	4500	4.5	2.8	46
		R	4500	4.5	0.0	
8	195	L	4200	4.0	2.3	60
		R	4200	4.0	0.4	

*Current amplitude was normalized to that current required for the full recruitment of the α component of the ACAP and expressed as × full α. We were unable to achieve 4.5 (our target) on every animal due to the current limitations of the stimulations.

#Percent of myelinated fibers undergoing either early axonal degeneration (EAD) or advanced axonal degeneration (AAD).

demonstrated a segmental damage to the individual fibers, which often have normal-appearing segments distal or proximal to the EAD foci (Fig. 2C).

At the ultrastructural level, EAD is seen to consist of collapsed myelin impinging upon the axoplasm. In some instances myelin ovoid bodies were present. Another prominent feature at seven days after stimulation, was a notable convolution or flattening of the myelinated axons sometimes associated with EAD, i.e., a departure from the normal oval or round configuration. Finally, at this interval after stimulation,

there was no phagocytic activity (by either macrophages or Schwann cells) nor was there any remyelinating activity.

At 21-28 days after the eight hour continuous stimulation (4.5 x full α) there was occasional focal endoneurial edema (Figs. 3A and 3B). At this stage, macrophage activity was present, and markedly fewer profiles were undergoing advanced axonal degeneration (AAD) compared to the number undergoing EAD at 7 days after stimulation. In addition, the myelin of the fibers undergoing AAD were condensed and often surrounded by a halo (Fig. 4A) which was confirmed by electron microscopy to be a portion of a large vacuole within the macrophage (Fig. 4B). In most cases, the axoplasm of these degenerating axons was essentially obliterated. Rare instances of remyelination were observed at this stage (Fig. 4A).

By 36-46 days following stimulation, there was a marked diminution of axonal degeneration. The few abnormalities remaining at this stage consisted of macrophages containing multilamellated myelin remnants (Fig. 5A) or cytoplasmic lipid inclusions (Fig. 5B). Even at 60 days following stimulation, sparse distribution of macrophages with phagocytosed remnants of axons in advanced stages of degeneration remained. Evidence of remyelination was also rare. Examination of the spinal cord at the light microscope level at the various periods following stimulation showed only a few degenerating axons in the dorsal and ventral funiculi. No chromatolysis or other abnormalities were present in the grey matter (Figs. 7A and 7B).

Morphometric Results Fig. 8A is a scatter plot of 2,009 fiber profiles from an unstimulated cat sciatic nerve. Each small circle corresponds to one myelinated fiber. The abscissa is the cross-sectional area of the myelin and the ordinate is the cross-sectional area of the axoplasm. Virtually all of the points lie between the SAP and hypomyelination lines; one fiber straddles the hypomyelination line and two points are in the SAP region. The latter correspond to fibers sectioned through the region adjacent to a node of Ranvier (the perinodal region).

Fig. 8B is a scatter plot of the fibers from a region at the periphery of a nerve that had sustained mechanical injury during or after implantation of the electrode array. The nerve had not been stimulated. The cat was sacrificed 112 days after implantation of the array. The points are more dispersed than in the normal nerve, and many lie in the hypomyelination region, indicating that their myelin sheaths are thinner than any in the normal nerve. The thin myelin indicates that the axons are regenerating and are being remyelinated, since the myelin encasing such axons remains thin permanently (Oishi et al, 1995).

Fig. 8C-8E are scatter plots of the fibers from the sciatic nerve of cats sacrificed 7 days after the nerve was stimulated for 8 hours at 50 Hz and at 4 α units. Many (up to 4.6%) of the points are in the SAP region, and nearly all of these represent fibers undergoing early axonal degeneration (EAD). (Compare with Figs. 2A-C.) By 21-28 days after a damaging stimulation (Fig. 8F), a few degenerating axons are still prevalent, but there are few thinly-myelinated axons that would indicate regeneration and remyelination of the damaged axons (at least not at the level of the electrode). (Compare with Figs. 4A and 4B). Interestingly, there were a significant number of degenerating axons in both nerves from the only cat that was allowed to survive for 60 days after receiving an intense stimulation (4.0 α units). This is depicted in Fig. 8G. Since these fibers were distributed uniformly through the cross-section of the nerve, they apparently are not the result of mechanical damage inflicted by the electrode matrix; this type of injury tends to occur at the periphery of the fascicles.

It is notable that the image analysis detected only 4 thinly-myelinated axons in 7 nerves from cats sacrificed 20-60 days after receiving a damaging stimulation. This indicates that few of the axons damaged by the electrical stimulation were regenerating or remyelinating.

DISCUSSION

Stimulation of the sciatic nerve for 8 hours at 3.0-4.5× α (one α unit is the current required for complete recruitment of the α component of the compound action potential) resulted in EAD of as many as 4.6% of the myelinated fibers at 7 days after the stimulation. There was good correlation between the histologic findings and quantitation of neural damage by morphometric techniques as presented in Table I. These results suggest that most of the EAD profiles represented fibers that were irreversibly damaged as indicated by the extensive phagocytosis between 7 and 28 days, yet phagocytosis by macrophages was present even at 60 days following stimulation. It is unclear if the degenerating axons 60 days after the stimulation are a delayed pathologic response to the electrical stimulation or represent delayed or ongoing phagocytosis by macrophages. At 60 days, the only elements of AAD were myelin breakdown products within foamy macrophages. As shown by both histologic and morphometric techniques, there did not appear to be a corresponding rate of regeneration (remyelination) over the entire 60 days of study. In support of this, at 28 days poststimulation, degenerating, phagocytosed axon remnants were surrounded by significant areas of endoneurial collagen, indicating permanent loss of at least some axons. However, a few instances of remyelination were shown by both techniques, and it is possible that more remyelination would have occurred had the study been extended to longer intervals. There were only a few degenerating fibers in the spinal cord and spinal roots and no chromatolysis of neurons in the dorsal or ventral horns of the spinal cord.

The ultrastructural characteristics observed in response to excessive electrical stimulation, in this and our previous studies, indicate that the neuropathy produced is most probably a "metabolic" axonopathy resulting from excitotoxic injury following high frequency stimulation. The metabolic or toxic effect produced in the selectively vulnerable population of fibers of the nerve following intense stimulation could conceivably be due to substrate depletion (e.g., glucose) increased extraaxonal

potassium or other toxic products. The metabolic insult hypothesis is further supported by the observation that the degenerating axons of this study closely resemble those described following isoniazid (isonicotinylhydrazide INH) therapy used in the treatment of tuberculosis (Cavanagh, 1973). Rats given isoniazid for 7 days show a pattern of axonal degeneration strikingly similar to that observed in the cat sciatic nerves of this study (Jacobs et al, 1979).

In summary, following high frequency, high pulse amplitude, continuous stimulation of the sciatic nerve for eight hours, some of the axons are irreversibly damaged, and degenerating axonal profiles (mostly undergoing phagocytosis) persist for as long as 60 days following the stimulation. There was very little regeneration within the time course of the study. We emphasize that the neural damage observed in this study was deliberately produced by the use of high frequency, high amplitude stimulation for the purpose of studying the mechanisms involved in neural damage. We have previously demonstrated that nerves can be safety and effectively excited at a low frequency even at high pulse amplitudes (Agnew et al, 1989, 1990, McCreery et al, 1992, 1995).

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WORK NEXT QUARTER

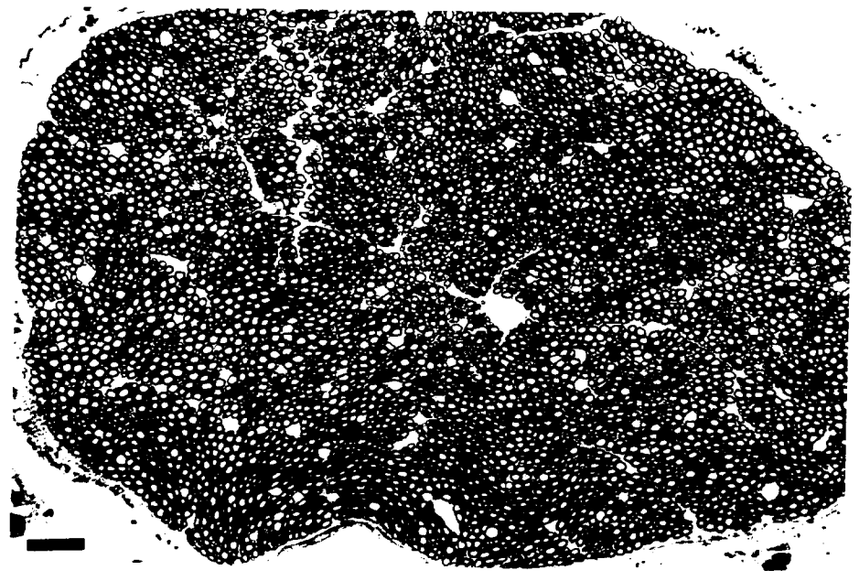
In the next quarterly report, we will present recent refinements of our fabrication procedures for intracortical activated iridium microelectrodes.

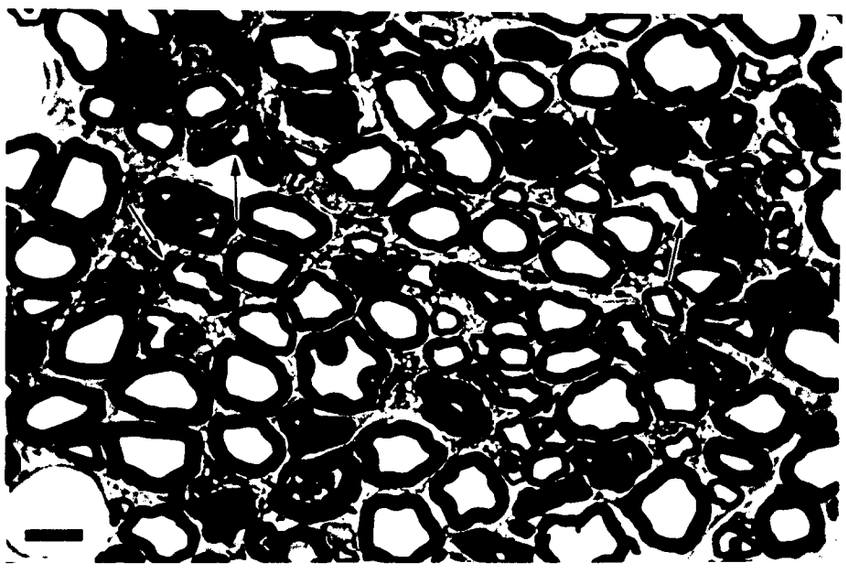
Fig. 1. Representative appearance of the bidirectional helical stimulating electrode array on the right sciatic nerve after partial removal of the overlying connective tissue sheath. Suture (arrowheads) secured the cables to the epineurium. The platinum band electrodes (arrows) are closely fitting but are not constrictive.

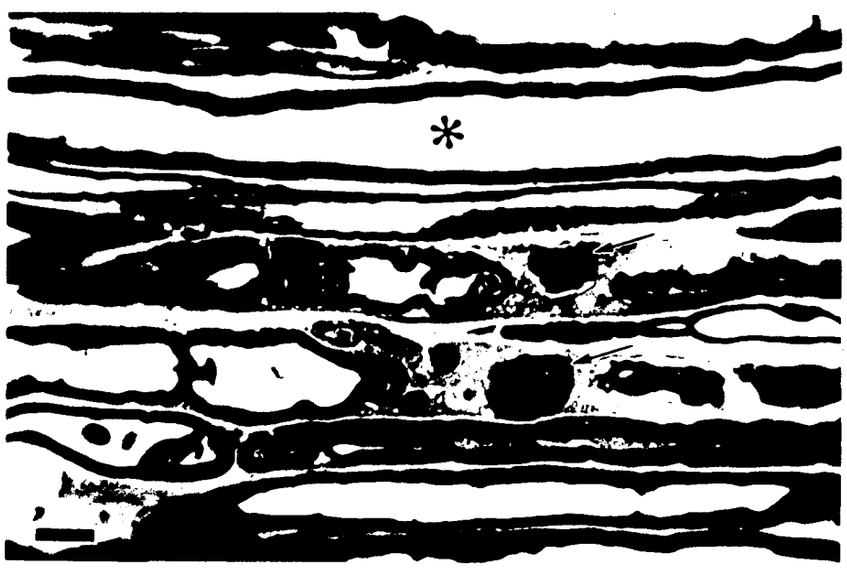
Fig.2 A. Animal PN-176. One µm-thick plastic section of the common peroneal component of the left sciatic nerve 7 days after 8 hours of continuous electrical stimulation using 3 × full α. Notice the uniform "salt and pepper" appearance of the axons throughout the fascicles. Although a few dark axons are undoubtedly perinodal segments, most are EAD (early axonal degeneration) profiles (see Fig. B, below). Bar = 100 µm.

B. Same fascicle as that shown in Fig. A, above. Several EAD profiles (arrows) show the narrowed, distorted shapes of axons undergoing the earliest stages of damage. Other dark axons show the markedly redundant myelin which, in some instances, has obliterated the axoplasm. Bar = 10 µm.

C. Same fascicle as that shown in A and B above. This longitudinal section shows the "myelin ovoid bodies" (arrows) typically seen in Wallerian degeneration and resulting from severe disruption and degeneration of the myelin. Note the normal-appearing fiber (asterisk). Bar = 10 µm. These and all subsequent light micrographs are taken from 1 µm thick plastic sections stained with Toluidine Blue and Azure II. The electron micrographs were stained with uranyl acetate and lead citrate.







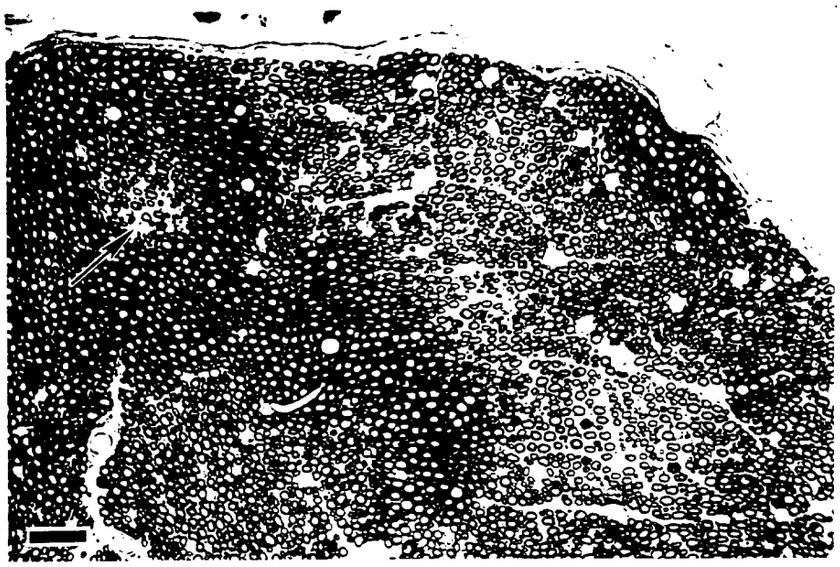
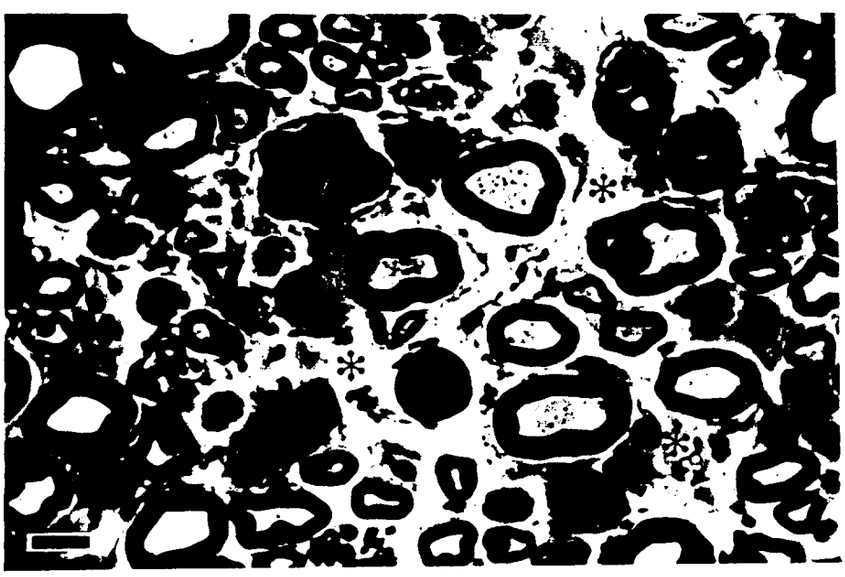


Fig. 3 A. Animal PN-188. Left sciatic nerve 21 days after continuous pulsing for 8 hours at 4.5 × full α. Note the (pale) areas of interstitial edema resulting in separation of axons. The arrow indicates one edematous area shown at higher magnification in B below. Bar = 100 μm.



B. Separation of axons caused by endoneurial edema typified by "structureless spaces" (asterisks). These spaces, lacking endoneurial collagen, distinguish interstitial edema from axonal loss characterized by their replacement with collagen. Bar = 10 μm.

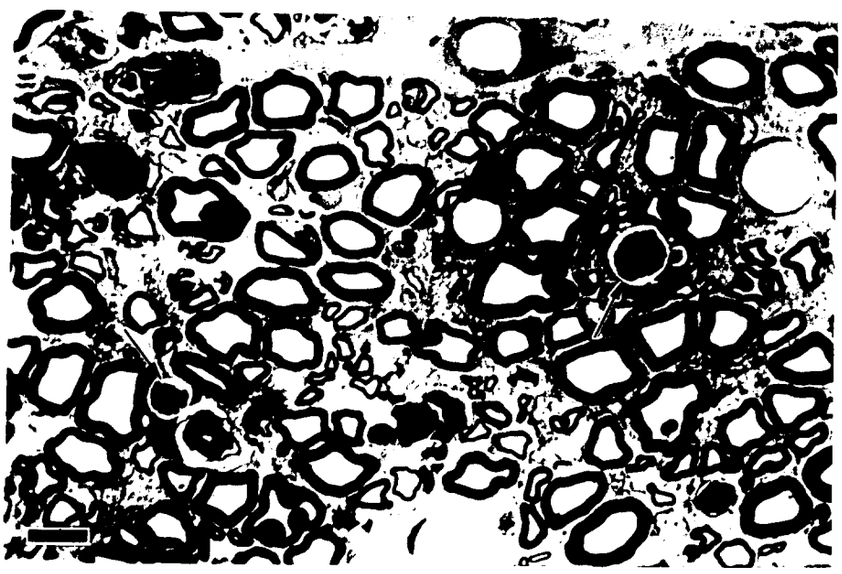


Fig. 4 A. Animal PN-189. Sciatic nerve 28 days after pulsing continuously for 8 hours with 4.5 × full α. EAD profiles are surrounded by a narrow halo (arrows) which, at the ultrastructural level, was shown to be the intracytoplasmic spaces of macrophages (see B below). Only a few remyelinating axons are present (arrowheads). The collagen-filled endoneurial spaces (collagen not distinguishable in the micrograph) indicate the complete loss of some axons.
Bar = 10 μm.

Fig. 4 B. Electron micrograph of same nerve as that shown in A. The macrophage in the center of the micrograph contains an engulfed axon and numerous lipid droplets, presumably originating from digested myelin. The identity of the macrophage was verified by the typical appearance of the nucleus and absence of a basement membrane. Presumably, a few adjacent axons (asterisks) show slight damage, as evidenced by their distorted profiles and departure from circularity. Bar = 2 μm.

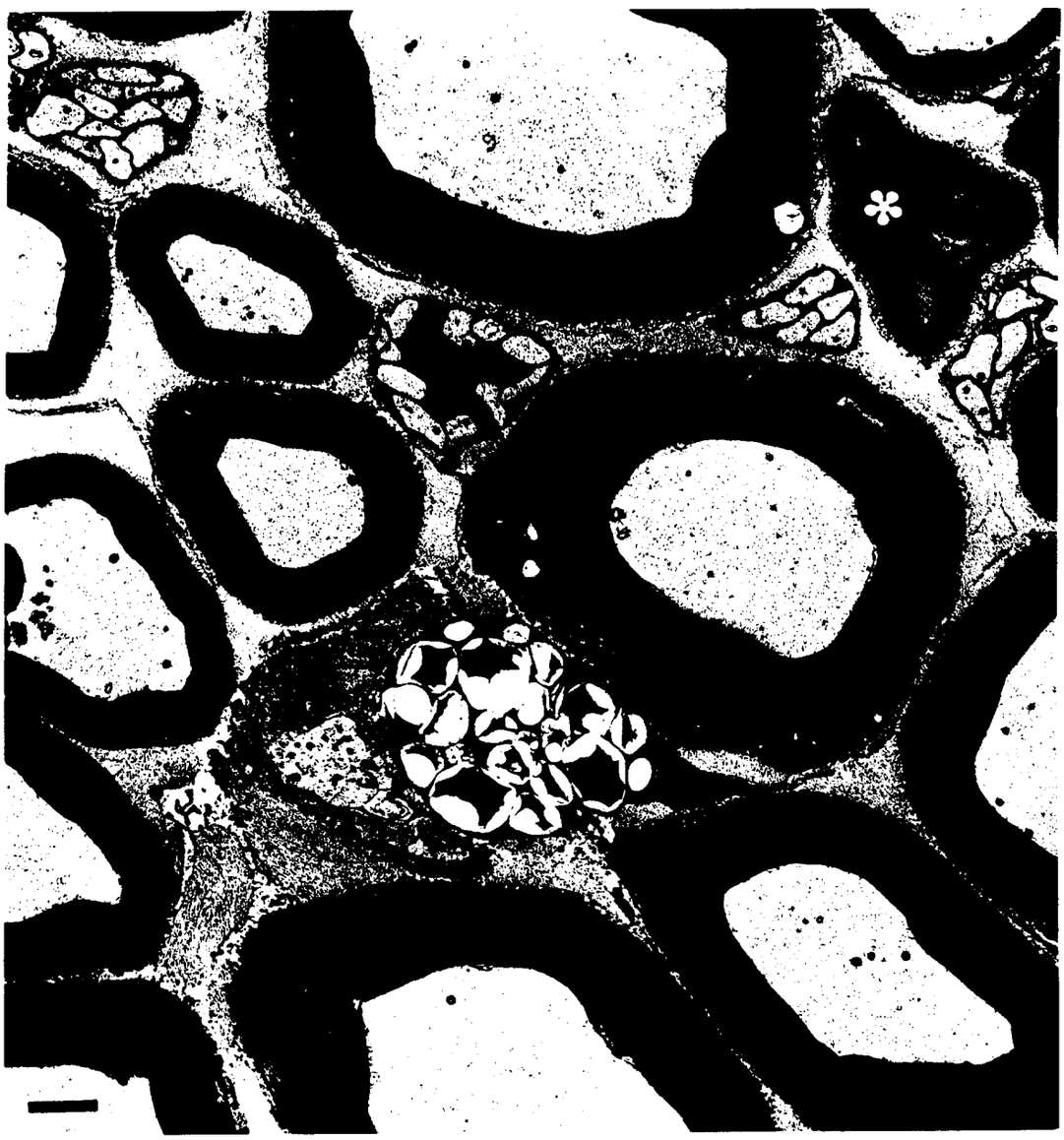


Fig. 5. Animal PN-192. Sciatic nerve 36 days following 8 hours of continuous stimulation with 4.5 × full α. In the center of the micrograph, a macrophage contains numerous lipid droplets, presumably the remnants of previously engulfed myelin fragments. Adjacent axons, both myelinated and unmyelinated, appear normal. Only moderate numbers of these macrophages, containing myelin or lipid, were still present at this time period. A nearby axon probably is slightly damaged as evidenced by its marked departure from circularity and dark axoplasm (asterisk). Bar = 2 μm.

Fig. 6. Animal PN-179. Sciatic nerve 46 days after stimulating continuously with 4.5 × full α. Electron micrograph showing one of a relatively few remaining phagocytosed degenerating axons at this late interval. In the center of the micrograph, a thin rim of macrophage cytoplasm surrounds the degenerating, fraying axon. The absence of a basement membrane rules out a Schwann cell identity for this engulfing cell and identifies it as a macrophage. Bar = 2 μm.

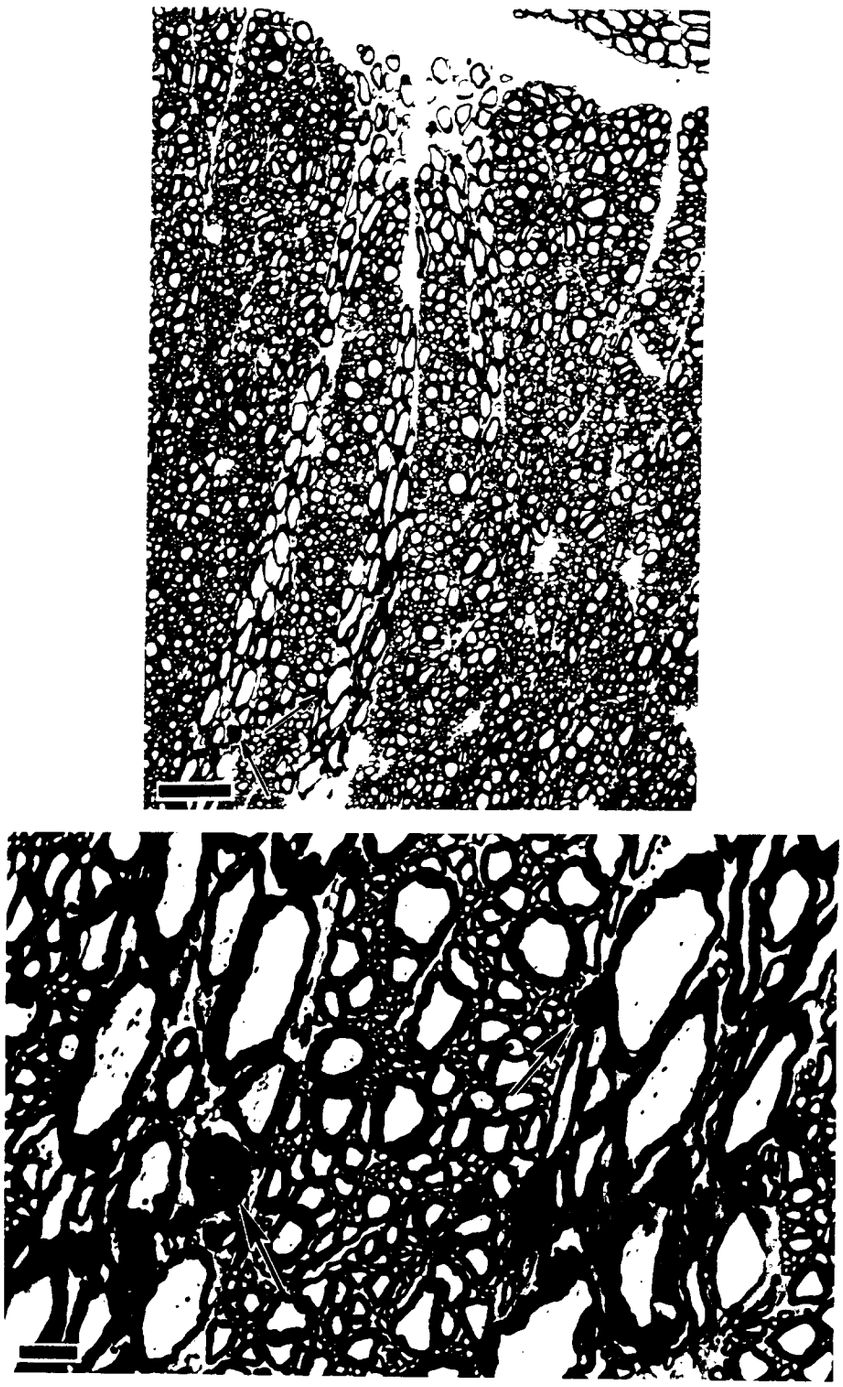


Fig. 7 A. Animal PN-189. Left dorsal funiculus of the spinal cord ipsilateral to the stimulated sciatic nerve shown in Figs. 4A and B. EAD was rare and never exceeded 3 profiles in any funiculus. This funiculus contains 2 EAD (arrows) originating in the afferent nerve rootlets entering the funiculus. A portion of a nerve rootlet lies in the right upper corner of the micrograph. Bar = 50 µm.

B. Same EAD profiles (arrows) as those shown above. All other axons appear normal. Bar = 10 µm.

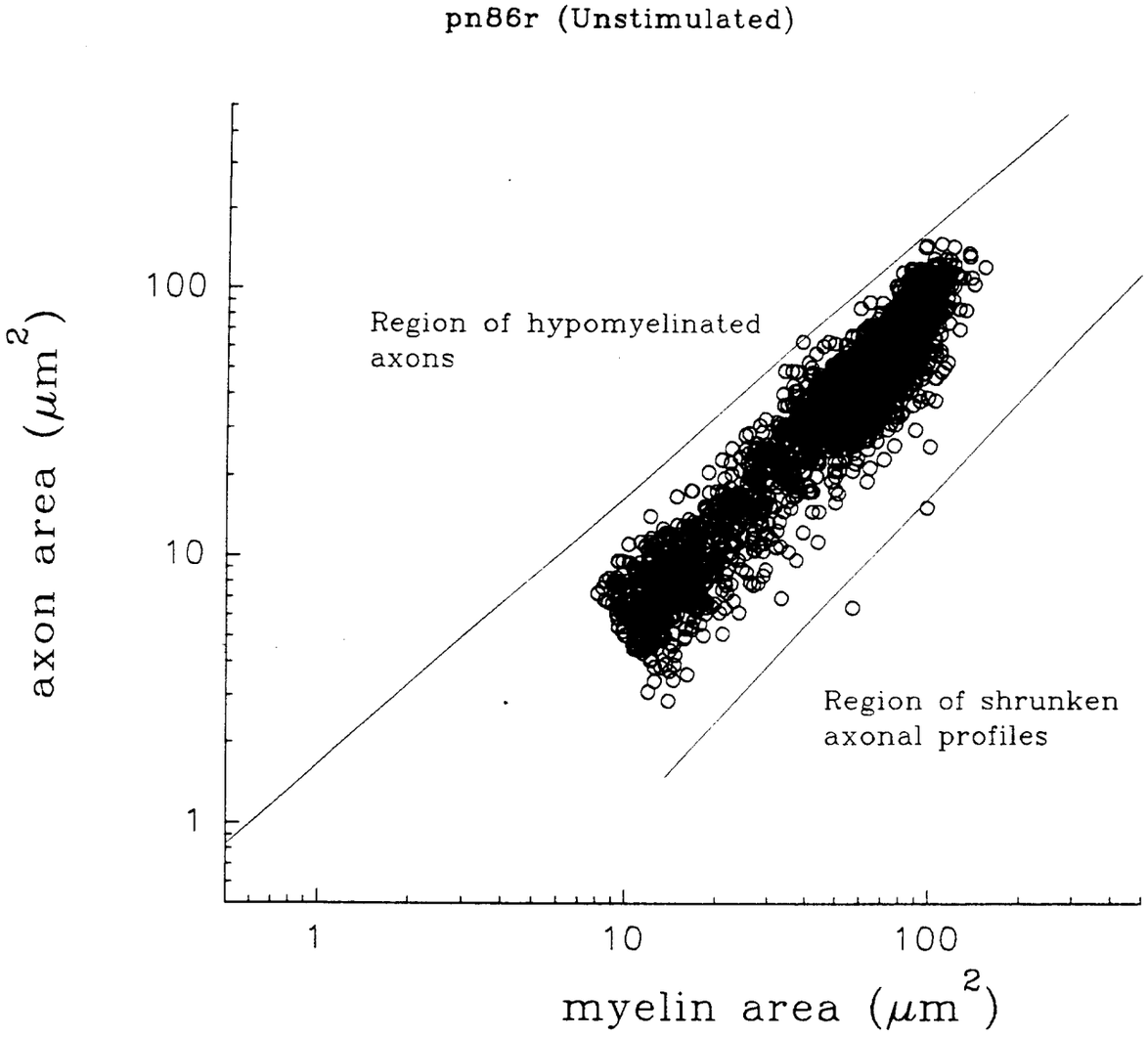


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Figure 8A

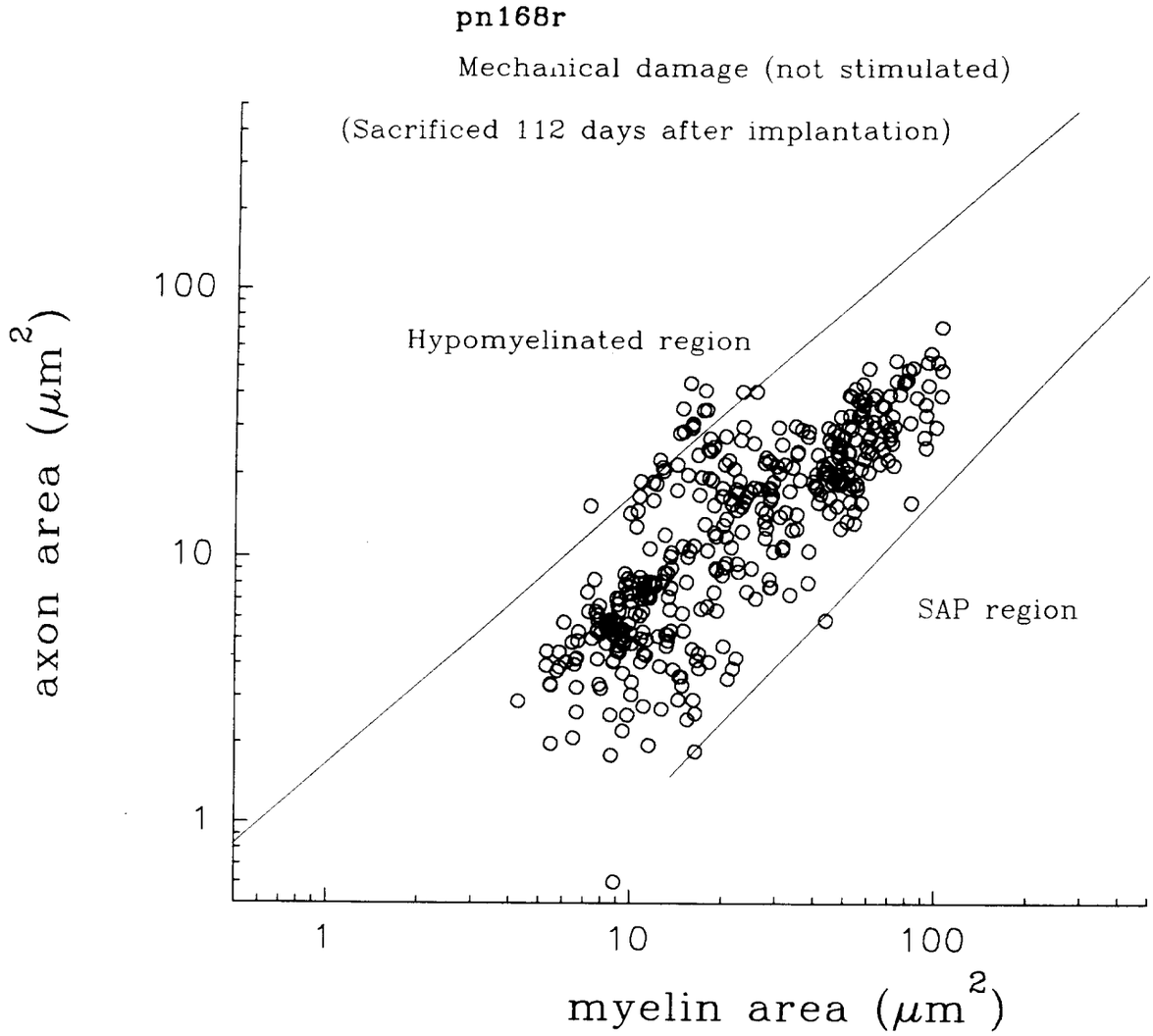

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Figure 8B

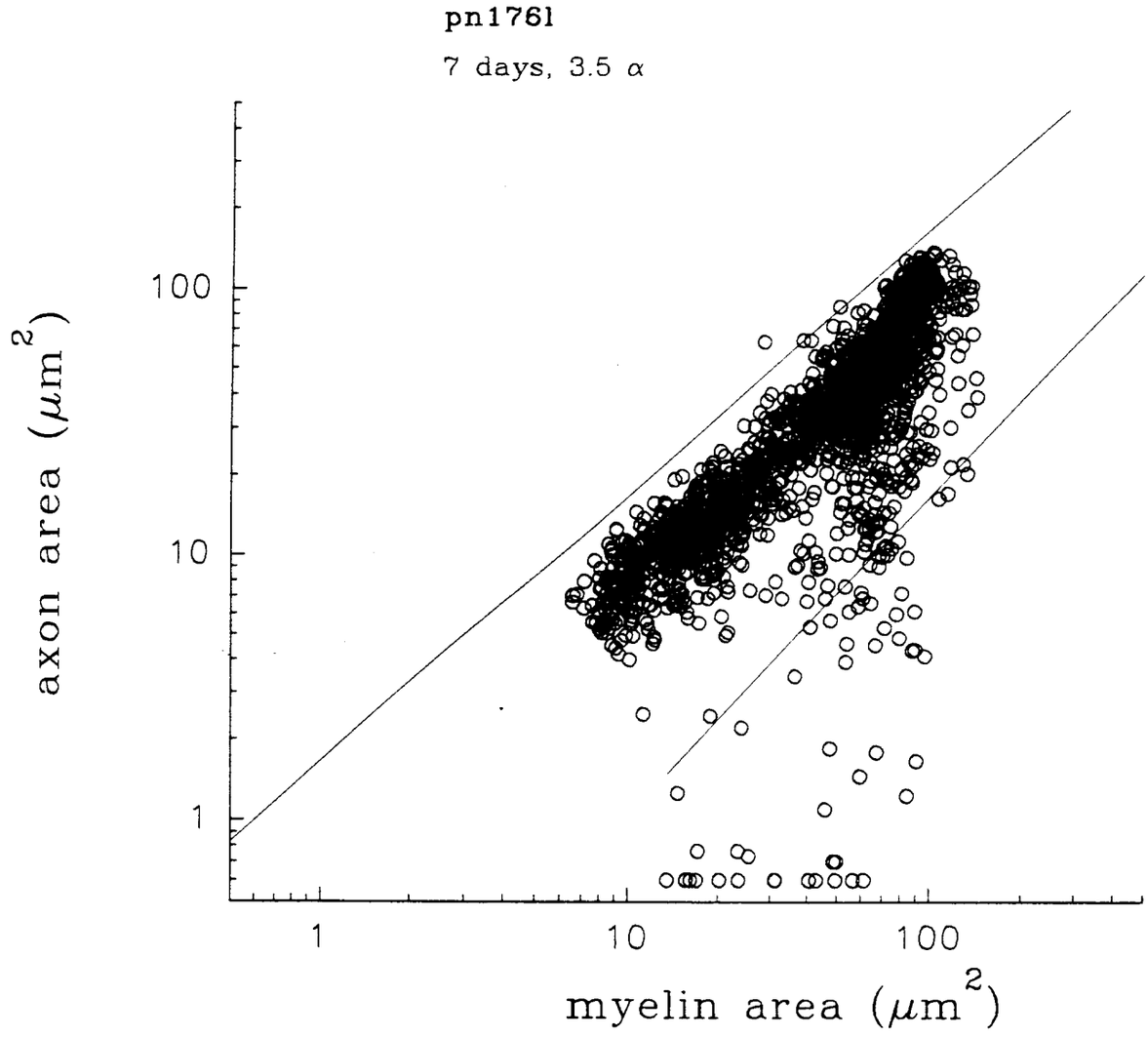

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Figure 8C

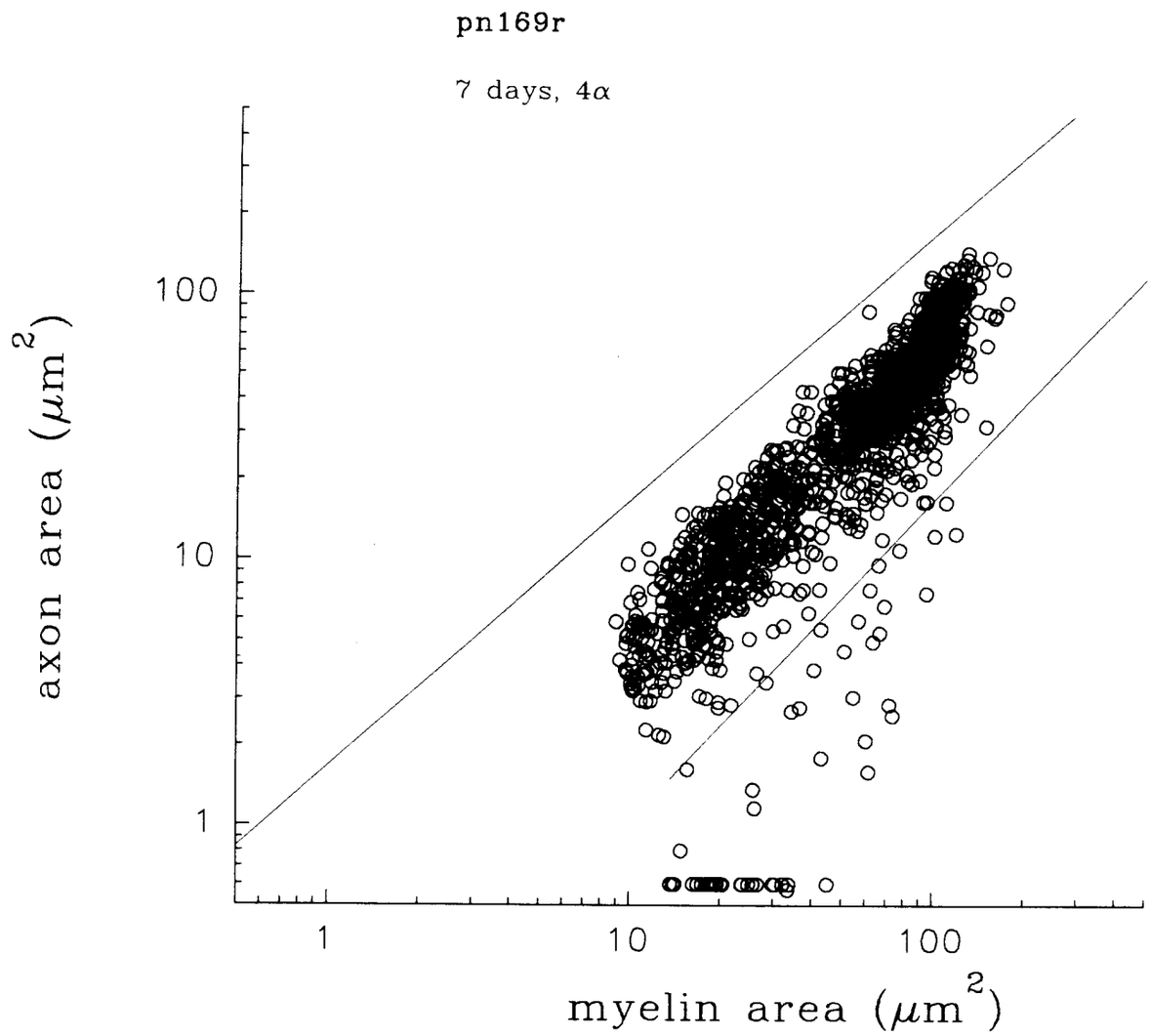

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Figure 8D

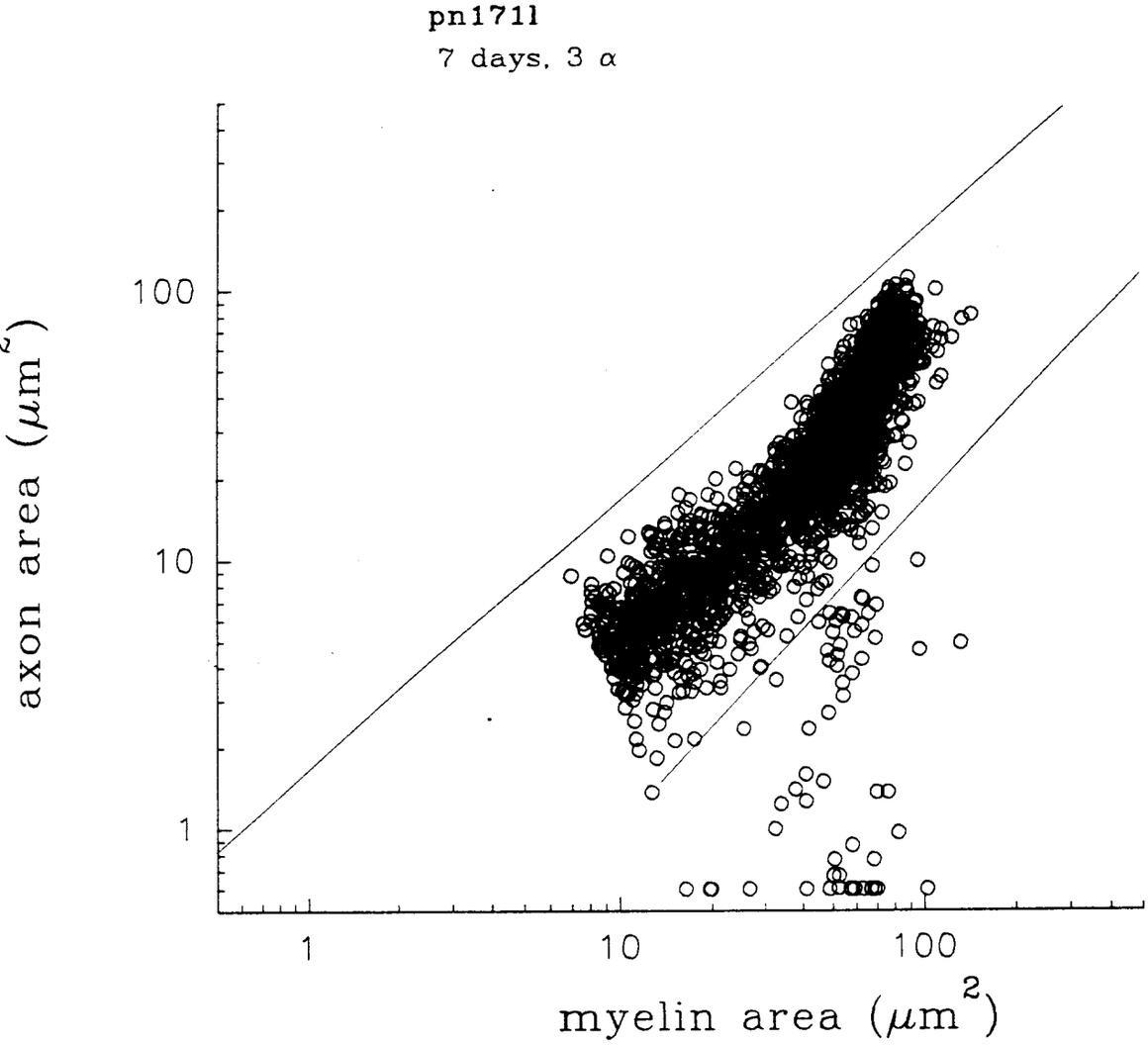

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Figure 8E

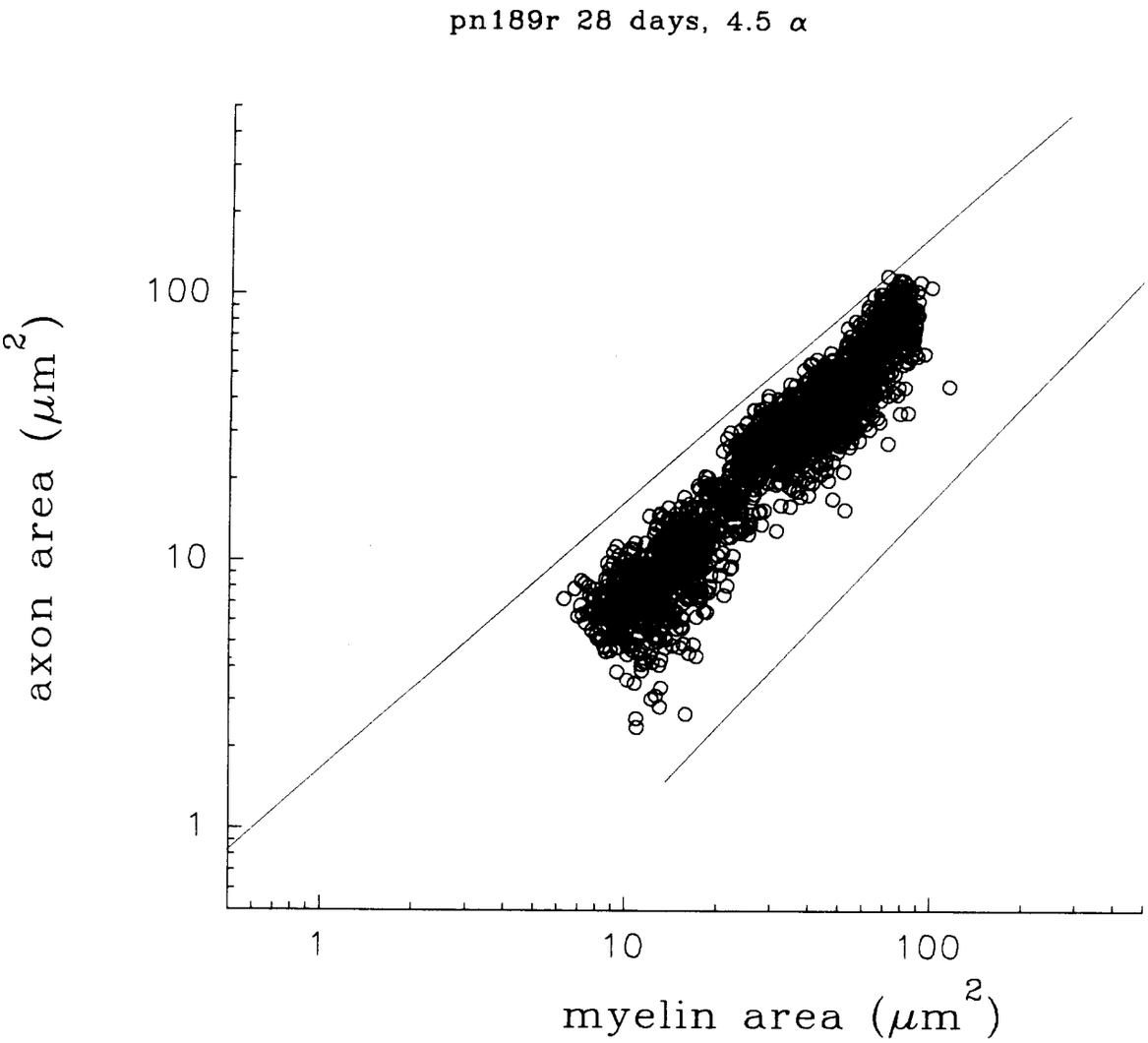

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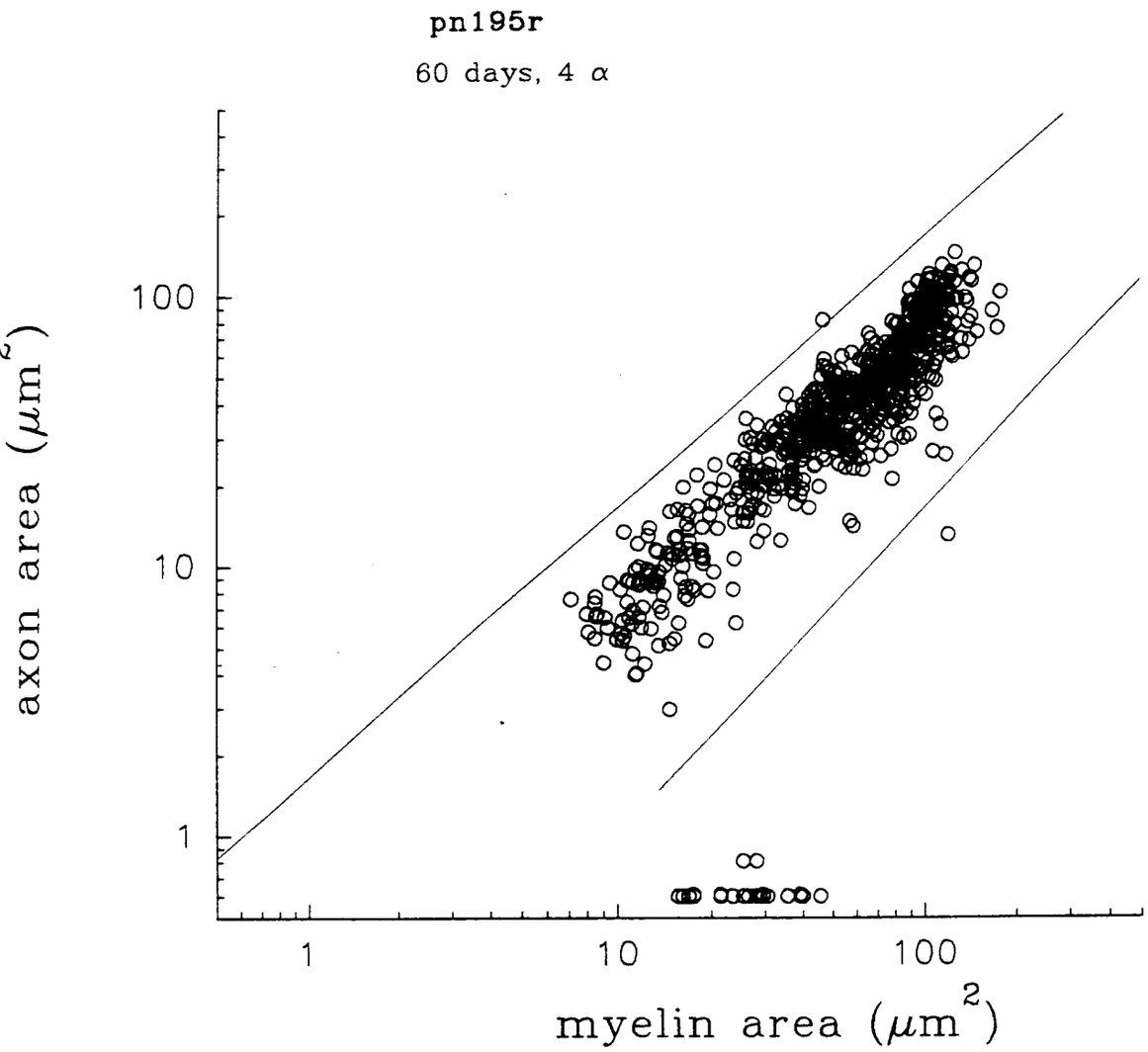
Figure 8F



d:edc\pn195t/195r.spg

Figure 8G